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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number: WO 92/21973
G01N 33/53, 33/543	A1	(43) International Publication Date: 10 December 1992 (10.12.92)

(21) International Application Number: PCT/SE92/00386

(22) International Filing Date: 5 June 1992 (05.06.92)

(30) Priority data:
9101735-0 7 June 1991 (07.06.91) SE

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: METHOD AND SENSOR MEANS FOR DETERMINING MYOCARDIAL INFARCTION MARKERS

(57) Abstract

A method of determining myocardial infarction analytes comprises (i) simultaneously determining from a blood, serum or plasma sample from a patient at least two different myocardial infarction analytes by contacting the sample with a flow cell sensor surface area or areas (3) supporting specific analyte binding ligands (8), and detecting any binding interactions at the sensor surface (3); (ii) removing bound analytes from the sensor surface (3); (iii) repeating steps (i) - (ii) for at least a second sample taken at a determined time interval from the first sample; and (iv) determining from the results obtained the variation with time of the cardiac analytes. A sensor means (2, 3) for use in the method has removably coupled to its sensing area(s) (3) at least two different ligands (8) specific for myocardial infarction analytes.

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METHOD AND SENSOR MEANS FOR DETERMINING MYOCARDIAL INFARCTION MARKERS

FIELD OF THE INVENTION

This invention relates to a method of simultaneously assessing from blood samples the variation with time of at least two different and independent markers indicative of myocardial damage to provide for early and reliable diagnosis of myocardial infarction as well as to improve the monitoring of the treatment and recovery after a myocardial event. The invention also relates to a sensor unit to be used in the method.

BACKGROUND OF THE INVENTION AND PRIOR ART

Acute myocardial infarction (AMI) is a major cause of deaths in the developed countries, the medical term describing the development of ischemia and necrosis of a portion of the myocardium. The ischemia is caused by occlusion of the artery by a thrombus preventing normal blood flow to the affected area of the myocardium and eventually resulting in tissue necrosis.

A number of serious complications like pulmonary edema, hypertension, thromboembolism, and ruptures of the heart can occur in AMI. Correct diagnosis and appropriate treatment at an early stage increases the chance of survival and diminishes the risk of complications. It is therefore essential that patients with suspected AMI are immediately sent to hospital for diagnosis and treatment.

The current clinical diagnosis of AMI is based on chest pain (sudden onset of pain which sustains for more than 15 minutes), electrocardiogram (ECG) and levels and kinetics of heart muscle specific enzymes and proteins. While chest pain and observed ECG changes may be sufficient for a safe diagnosis in some cases, they are, however, often of a rather nonspecific nature, and the final confirmation of AMI and differentiation from other cardiac or non-cardiac events can for a large group of patients only be made by monitoring the mentioned biochemical parameters by blood sampling at regular intervals, usually

6 to 18 hours between sampling, and analysis at centralized laboratories. Today a patient with suspected AMI therefore may spend from a few hours to more than 48 hours at the coronary or intensive care unit before a reliable diagnosis can be made, depending on the individual patient and the readiness of the physician.

It is, of course, desirable that the correct diagnosis could be made in all cases at an earlier stage. On one hand, in case of an AMI, the early intervention with appropriate treatment with thrombolytics would reduce the mortality and diminish the risk of complications. In fact, thrombolytics have to be used within the first six hours from the onset of pain to be beneficial, the mortality being substantially reduced the earlier the treatment is established (around 10% per hour). On the other hand, the relatively larger group of patients (about two thirds) not suffering from AMI could be excluded from expensive, inappropriate and sometimes fatal therapy, other causes of the AMI-like symptoms being e.g. angina pectoris or pain from the gastro-enterological tract. Thus, the treatment with thrombolytics has severe side effects such as stroke and haemorrhagic bleedings. Today up to around 2% of the patients treated with the current thrombolytics, tissue plasminogen activator (tPA) and streptokinase, are hit with these side effects. Accurate diagnosis is thus fundamental in these cases. Also, the great economic importance of such early identification of the patients not requiring the AMI treatment is readily appreciated, considering the substantial cost reductions obtained when such patients after a much shorter time than before may be transferred to an ordinary medical ward or in the most favourable cases can be sent home.

In the treatment of AMI by thrombolytics the objective of the treatment is to achieve reperfusion of the blocked coronary artery before the tissue has got an irreversible damage. It is known that the cardiac enzymes show different patterns if a reperfusion is achieved or not. It is a matter of course that a real-time analysis permitting

bedside monitoring of the patients would be desired. So far this has not been feasible with today's assay methods and technology.

Similarly, it would, of course, also be desirable to be able to continuously monitor cardiac enzymes for detecting a possible AMI initiated during thorax surgery before closing the thorax.

Current serum assays are based on the leakage of intra-cellular enzymes or other proteins from the damaged or necrotic cells which result from the myocardial infarction. The recognized standard assay for the detection of a heart attack has for a long time been the creatine kinase (CK) test. This test involves the determination of CK and its isoenzyme CK-MB which exhibit detectable peak concentrations around twelve hours after the myocardial event has occurred. Another conventional protein tested is lactate dehydrogenase (LD) and its isoenzymes, and in recent times the determination of noncatalytic proteins like myoglobin and troponin T has been rendered possible as a result of sensitive immunological determination methods. Still another protein known to be released from cardiac muscle following myocardial infarction and forming the basis of a test is myosin light chain. Each one of the mentioned marker proteins exhibits specific serum concentration changes corresponding to the kinetics and release of the respective protein in an acute infarction.

The combined analysis of two markers has been suggested to improve the recognition of myocardial infarction. Thus, combined analysis of CK and LD is described in e.g. Limbird, Lee, Diss. Abstr. Int. B 1974, 34(11), 5322-3.

WO91/01498 discloses the simultaneous or sequential testing for creatine kinase and myosin light chain for the early detection of a myocardial infarction. While the combination of the two assays is said to provide a very reliable diagnostic test for the early phase of a heart attack, it also permits differentiation between a myocardial infarction and other ischemic events causing

cardiac pain, such as angina pectoris. The latter feature is due to the fact that myosin light chain is released not only in case of an acute myocardial infarction, but also for other cardiac injuries, whereas creatine kinase is substantially only released in the instance of an acute myocardial infarction.

While the dual combination assays of the type described above offer a more accurate and reliable diagnosis, they suffer from the basic disadvantage of being based on conventional type assays, usually solid-phase enzyme immunoassays, which have to be performed at a laboratory and usually cause a considerable time to pass before the final diagnosis can be made, as mentioned above.

There is therefore a need of an assay procedure which permits a reliable diagnosis of a possible myocardial infarction to be obtained in a shorter time, which in its entirety may be performed at or in the close vicinity of the patient, and which may easily be automated.

SUMMARY OF THE INVENTION

One object of the present invention is therefore to provide an assay method by which it is possible to reliably diagnose AMI in such a short time that a treatment with thrombolytics will be beneficial.

Another object of the invention is to provide an assay method by which it is possible to rapidly and reliably exclude AMI to thereby reduce care costs in that such patients within a short time may be transferred to an ordinary (substantially less costly) ward or even be sent home.

Still another object of the invention is to provide an assay method by which it is possible to efficiently monitor the treatment of an AMI with thrombolytics such that the treatment may be immediately interrupted as soon as reperfusion has been obtained, or the patient may be subjected to an alternative treatment in case reperfusion is not obtained in a reasonable time.

Another object of the invention is to provide an assay method for the diagnosis of AMI or monitoring of treatment

with thrombolytics that may be performed at or close to the patient's bedside.

Still another object of the invention is to provide an assay method for monitoring cardiac enzymes to detect the possible initiation of an AMI during thorax surgery, and which may be performed in the operating room.

A further object of the invention is to provide a sensor means for use in the assay methods mentioned above.

The above mentioned objects are achieved with the method and sensor means of the present invention. A basic concept of the invention resides in determining with short intervals the variation with time of at least two, preferably at least three different markers, i.e. enzymes or proteins, of myocardial infarction in real-time measurements in a flow cell system, which measurements are based upon interactions of the analytes with ligands bound to a sensor surface, the interactions causing a detectable change of the physico-chemical characteristics of the surface. While the study of at least two different cardiac analytes or markers will provide sufficient information for insuring an early diagnosis, the use of sensor surface technology to detect analyte-ligand interactions provides for real-time analytical procedures which are sufficiently rapid for permitting the analysis of blood samples taken at short intervals and which also are apt to automation and need not be performed at a laboratory, but may readily be performed at the patient's bedside or in an operating room.

It is to be noted that the terms "different markers" or "different analytes" as used herein mean markers or analytes originating from different and discrete genomes. Therefore, any post-translational modifications like glycosylation or fragmentation of macromolecules do not qualify them as being regarded as "different" analytes. For example, CK-MM_A and CK-MM_B are not different analytes in the context of the present invention.

Thus, in accordance with one aspect of the invention there is provided a method of AMI marker determination or monitoring, comprising the steps of

(i) simultaneously determining from a first blood, serum or plasma sample from a patient at least two, preferably at least three different analytes indicative of myocardial infarction by contacting in a flow cell or cells the sample with one or more sensor surface areas each supporting a different ligand or mixture of different ligands capable of specifically binding to a respective analyte, and, optionally after additionally binding an analyte specific reagent or reagent complex to the bound analytes, detecting any binding interaction of each analyte with its ligand as a consequential change of the physico-chemical characteristics of the sensor surface;

(ii) removing each bound analyte from its sensor surface bound ligand by passing a regenerating liquid through the flow cell;

(iii) repeating step (i) for at least a second blood, serum or plasma sample taken from the patient at a determined time interval from said first sample; and

(iv) determining from the results of steps (i) to (iii) the variation with time of said cardiac analytes.

By the term "reagent complex" as used herein is meant that the reagent is bound to one ore more other species. Such a reagent complex may be added as an assembly or be formed successively after the reagent has bound to the analyte as will be further elucidated below.

In accordance with another aspect of the invention there is provided a sensor means comprising, immobilized to one or more sensing areas thereof, either individually or in combination, at least two, preferably at least three different ligands, each ligand being capable of specifically binding to a respective analyte indicative of myocardial infarction, said sensor means being adapted for the detection of any analyte-ligand interaction as a consequential change of the physico-chemical characteristics of the sensing surface, and said ligand supporting surface areas being regeneratable after the coupling of analytes thereto.

The different ligands may thus be either individually immobilized to respective sensing areas, or co-immobilized to a single sensing area. Combinations of these embodiments are, of course, also possible, i.e. that there are two or more sensing areas, each area supporting co-immobilized ligands.

In a preferred embodiment the sensor surface is a surface capable of exhibiting surface plasmon resonance (SPR) and the detection of the cardiac analytes or markers is carried out by surface plasmon resonance spectrometry.

BRIEF DESCRIPTION OF THE DRAWINGS

Other objects and advantages of the invention will be apparent to those skilled in the art from the following detailed description thereof taken in conjunction with the accompanying drawings. In the drawings:

Fig. 1 is a schematic illustration of a per se known SPR-based flow cell measurement system;

Fig. 2 is an exploded sectional partial view of a flow cell unit useful for the purposes of the present invention;

Fig. 3 is an SPR-sensor diagram showing the co-immobilization of a monoclonal antibody specific for CK-MB and a monoclonal antibody specific for myoglobin to a sensing surface;

Fig. 4 is a corresponding diagram as in Fig. 3 showing the analysis of a sample containing elevated levels of CK-MB and myoglobin using the sensing surface with immobilized CK-MB and myoglobin monoclonals in Fig. 3; and

Fig. 5 is a corresponding diagram as in Fig. 4 showing the analysis of a sample not containing CK-MB and only a normal level of myoglobin.

DETAILED DESCRIPTION OF THE INVENTION

As mentioned above one basic feature of the invention resides in the simultaneous measurement of several, viz. at least two, preferably, however, at least three, or even e.g. four or five, different myocardial infarction markers or analytes. While the diagnostic sensitivity and specificity of such markers will vary, the appropriate selection of analytes and the determination thereof

simultaneously from blood samples taken at short intervals will provide for an analytical picture or pattern which will be very useful for diagnosing or excluding AMI. The relationship between the various analytes during a certain time interval will also give an indication of how long an infarction condition has been going on, for example based upon the rise of the level of one analyte and the decline of another. Likewise, the areas under the respective analyte graphs will indicate the extent of the damage to the cardiac muscle tissue.

The term "simultaneously" as used herein is to be understood in a rather broad sense meaning that the respective determinations of the analytes need not be started exactly simultaneously but that certain delays may be permitted as long as a comparison between the different analyte levels determined may be considered as meaningful.

Another basic feature of the invention lies in the already mentioned repeated analyses at, preferably, regular short intervals permitting the variation of time of the cardiac markers to be determined. Such information will considerably improve the possibilities of making a reliable AMI diagnosis or exclusion.

In combination the above basic features will permit a very reliable diagnosis or exclusion of AMI to be established at a desirably short time after the patient has been brought to hospital, or in the alternative, efficient monitoring of the treatment with thrombolytics as well as of thorax surgery.

An apparatus system enabling the desired type of analytical procedures to be performed should (i) rely on a measuring principle based upon the interaction of the analytes with ligands immobilized to a sensor surface and detecting complex formation as a consequential change of the physico-chemical properties of the sensor surface; (ii) have a flow cell system permitting the desired simultaneous detection of several analytes; and (iii) have a sensor surface or surfaces which can be regenerated in situ in the sense that bound analytes may be removed from the

respective ligands to permit consecutive analyses on one and the same sensor surface(s) after a regenerating step. All the requirements (i) to (iii) may be met by analytical technology which is known per se in the art.

In order to increase the above mentioned change of the physico-chemical properties of the sensor surface, further complexing of the bound analytes, and thereby an increase of the thickness of the bound substance layer, may be accomplished by reacting the ligand bound analytes with a secondary reagent specific to the analyte, i.e. a sandwich assay, as is per se known in the art. Such bound secondary reagent may then, if desired, be further complexed by reacting it with a tertiary reagent, etc. to still more increase the surface change to be detected.

In the case of optically based methods, and especially those based upon SPR spectroscopy, it is possible to increase the detected surface change further by labelling of the secondary and/or tertiary reagent, etc by an optically dense species, such as a particulate label, for example, glass, latex, colloidal silver or gold, metal oxide or ferritin; see e.g. EP-A-276 142.

By using a secondary reagent to perform a sandwich type assay on the sensor surface as outlined above, not only the sensitivity and in some cases the specificity will be increased, but also the dynamic measuring range may be extended in that the primary response may be used rather than the secondary response if the range of the latter would be insufficient.

The measuring principle as defined above will enable real-time measurements to be conducted and thereby permit the desired performance of consecutive analyses of blood samples taken with short intervals. Such a measuring principle, and the sensor surface associated therewith, is also readily adaptable to flow cell systems. Among methods relying on the said measuring principle may be mentioned internal reflectance methods, particularly evanescent wave spectroscopy (EWS), such as attenuated internal reflection (ATR) spectroscopy, total internal reflectance fluorescence

(TIRF) spectroscopy, and surface plasmon resonance (SPR) spectroscopy, or wave guide spectroscopy. All these methods are based upon the examination of an optical property of a solution bordering a surface where total internal reflection has occurred. Other types of sensors that may be used are those based upon photoacoustic piezoelectric, surface acoustic wave (SAW), or electrochemical measurements, etc. Particularly suitable for the purposes of the invention is SPR spectroscopy as will be described in more detail below.

A flow cell system is essential for providing a rapid and easy to handle system apt to automation. By the term "flow cell" (which is to be interpreted in a broad sense) is meant that the sample and other analytical fluids will flow past the sensing surface or surfaces at a constant rate. Such a system may, as mentioned above, in one embodiment thereof comprise a plurality of sensing surface areas, i.e. one for each cardiac analyte as well as a control area, and optionally one or more areas for other purposes as will be described below. The flow may be either in parallel or in series with respect to the different sensing surface areas. A parallel flow cell system may comprise several separate flow cells arranged in parallel, whereas in a series system several separate flow cells may be connected in series. A parallel or serial flow system, respectively, may also be formed by several defined sensing surface areas contained within a single flow cell. For an example of a flow cell system comprising several flow cells, it is referred to our published PCT-application WO 90/05295 (the full disclosure of which is incorporated by reference herein) disclosing a fluid handling block unit comprising a plurality of open channels for defining multiple flow cells together with a sensor plate applied thereto.

In another and novel embodiment of flow cell system, the ligands for all the cardiac analytes may, as already mentioned above, be co-immobilized in a single surface area. Thus, the specific response (Stenberg et al., J.

Colloid Interface Sci. 143 (1991) 513) for surface concentration detecting devices, such as surface plasmon resonance detection, is in principle (at least down to the diffraction limitations of the optical system) independent of the size of the detecting area. This is in contrast to absolute measuring devices such as ordinary solid phase assays where the specific response is area dependent.

Normally, the different immobilized capturing molecules are immobilized to different detection areas as described above. The independence of area size for the surface concentration detecting device will, however, in accordance with this novel and inventive concept, allow reduction in the number of detection areas by co-immobilization of two or more different capturing molecules onto the same area, the specificity then being revealed by sequential injections of second reagents (such as antibodies) specific for each analyte bound by the respective capturing molecules. As is readily understood, such a procedure will greatly reduce the complexity of the optical and mechanical design of the system for the concentration determination of a panel of analytes.

Sensor surfaces which may be regenerated as defined above a substantial number of times, e.g. 50 to 100 times, are known per se in the art and are, for example, described in our published PCT-application WO 90/05305 (the full disclosure of which is incorporated by reference herein). These sensor surfaces comprise a substrate coated with a metal film to which has been attached a layer of an organic polymer or a hydrogel forming a so-called basal surface which may contain functional groups for selectively binding the desired ligands. For further details on the production of such surfaces it is referred to our published PCT-application WO 90/05303 (the full disclosure of which is incorporated by reference herein). Such a surface is easily regeneratable in situ in a flow cell. Thus, after the determination of one sample is completed, the bound analytes can be removed from the respective ligands to prepare the sensing surface(s) for a subsequent assay of

another sample. This is made possible by the binding of the analyte to the ligand being broken by the regenerating fluid whereas the binding of the ligand to the sensor surface is not.

Exemplary of myocardial infarction analytes or markers from which the test panel may be selected are myoglobin, creatine kinase (CK) (also known as creatine phosphokinase) and its isoenzymes and isomers thereof, especially CK-MB, tropomyosin, lactate dehydrogenase (LD), troponin C, T and I, aspartate aminotransferase, and myosin, especially the light chain thereof.

Presently preferred infarction markers for the purposes of the present invention are CK-MB, myoglobin and troponin I.

However, none of the above mentioned markers is absolutely specific for acute cardiac muscle damage. For example, both CK-MB and myoglobin may also be present in skeletal muscle damages. It is therefore, as already mentioned above, preferable to simultaneously test for one or more control analytes. One example of such a control analyte is carbonic anhydrase III (CA III) which has been shown to be present in substantial amounts in skeletal muscle, in minor amounts in i.a. smooth muscle cells, but not at all in the myocardium. CA III can therefore be used to distinguish whether increased concentrations of e.g. myoglobin originate from cardiac or skeletal muscle (see e.g. Väänänen H.K. et al., Clin. Chem. 36/4, 635-638 (1990).

The ligands for the specifically binding cardiac analytes, such as those mentioned above, are usually antibodies, particularly monoclonal antibodies. By the term "antibodies" as used herein is also to be understood active antibody fragments, antibodies and fragments thereof produced by genetic engineering, etc.

The functionalizing of the basal sensor surface with the ligands is simplified if the ligands are chimeric molecules, i.e. comprise a common part for binding to the basal surface and a variable part for binding to the

different cardiac analytes. In the case that the sensing surface comprises a basal dextran layer, the chimeric molecule may consist of an antibody to dextran which is conjugated to a monoclonal directed against the desired cardiac analyte.

Antibodies that may be used as ligands are described in the prior art, and may also be produced by methods known per se, e.g. by hybridoma or recombinant DNA technology.

Thus, antibodies against creatine kinase and creatine kinase MB are, for example, described in EP-A-288 179, EP-A-339 814, EP-A-261 781, and US-A-4,912,033.

Antibodies against myosin light chain are, for instance, disclosed by WO 91/01498, WO 90/15329, and US-A-4,879,216.

Antibodies directed against troponin T are described in, for example, EP-A-394,819, and antibodies against troponin I are described by Cummins B. et al., Biochem. Soc. Trans. 15 (1987) 1060-61.

Myoglobin antibodies are, for instance, disclosed in JP-A-54011231.

Antibodies directed against lactate dehydrogenase are described in, for example, DE-A-2 350 711.

The preparation of antibodies directed against CA III are described by Väänänen H.K. et al., Histochemistry 1985; 83: 231-5, and by Kato K. et al., Clin. Chim. Acta 141 (1984) 169-177.

Several antibodies are also commercially available, such as monoclonals against CK-MB and myoglobin.

For the functionalization of the sensor surfaces with the ligands for the respective cardiac analytes it is referred to the aforementioned WO 90/050305.

The blood samples taken from the patient may be analyzed directly as whole blood, but it may be preferable to use plasma or serum prepared from the blood sample, e.g. as obtained by an initial centrifugation or filtration step.

With the above described method and sensor means of the present invention, using the mentioned analytical

apparatus, it is thus possible to in a short time, by taking blood samples at short intervals and using bedside apparatus and thereby dispensing with central laboratory determinations, diagnose or exclude, respectively, an AMI. Similarly, the treatment with a thrombolytic, as well as the state of the myocardium during heart surgery may readily be monitored, as already mentioned above.

A further beneficial feature of the method of the present invention is that it may also provide information about the presence in the blood samples of antibodies against streptokinase. Thus, whereas streptokinase is about tenfold cheaper than tissue plasminogen activator (tPA), tPA is necessary if the patient has a high level of antibodies against streptokinase or is allergic thereto. About 90% of the patients experiencing a reinfarcation within a year have neutralizing antibodies. Neutralizing antibodies can also occur after a flu, if the infection is caused by streptococcal bacteria. Therefore, the method of the invention preferably also comprises testing for such neutralizing streptokinase antibodies by including a streptokinase ligand (or an antibody against the anti-streptokinase antibody) in the or one of the sensing areas. Thereby such crucial information about the level of neutralizing streptokinase antibodies in a patient can be determined at the same time as the AMI diagnosis is performed.

In a preferred embodiment the present invention thus comprises determining (i) at least two, and preferably at least three, different infarction analytes; (II) one or optionally more control analytes; and (iii) streptokinase antibodies.

Hereinafter the invention will be described, by way of example only, with regard to a particular SPR-based embodiment, reference being made to Figs. 1 and 2 mentioned above. First, however, the surface plasmon resonance (SPR) phenomenon will be briefly explained.

If the angle of incidence of light directed towards an interface between two transparent media of different

refractive indices exceeds a critical angle, the light is reflected back into the medium having the higher refractive index, so-called total internal reflection. However, despite the total reflection an electromagnetic field component of the light called the "evanescent wave" penetrates a short distance (of the order of a wave length) into the medium of lower refractive index. If the interface between the media is coated with a thin metal film, such as silver or gold, and the light is plane-polarized and monochromatic, the evanescent wave will at a certain angle of incidence interact with collective electron oscillations, called plasmons, in the metal. This phenomenon - called surface plasmon resonance (SPR) - will be observed as an intensity dip in the reflected light. To couple the light to the interface such that SPR arises, two alternative arrangements are used, either a metallized diffraction grating (Woods effect) or a metallized glass prism or a prism in optical contact with a metallized glass substrate (Kretschmann effect). The specific angle of the occurrence of SPR is sensitive to refractive index changes close to the interface of the medium of the lower refractive index. Thus, if the high refractive index medium is glass and the medium of lower refractive index is an aqueous solution in contact with the glass, changes of the solution close to the interface, e.g. by the adsorption of a protein layer, will cause a corresponding shift of the resonance angle. SPR may therefore be used for detecting e.g. immunoassay reactions as is well known in the art.

A schematic illustration of an SPR based biosensor system of the flow cell type which is known in the art and may be used for the purposes of the invention is shown in Fig. 1. In the figure a flow channel 1 has an open top portion covered by a sensor plate or chip 2 of glass coated with a metal film 3, more specifically of gold, to define a flow cell. A prism 4 contacts the other side of the glass plate 2 to couple a wedge-shaped beam 5 of p-polarized light from a monochromatic light source 6 thereto. The reflected light is directed against a detection unit 7

comprising a matrix (i.e. rows and columns) of photodetectors. The gold film surface exposed to the fluid passing through flow cell 1 has ligands 8 immobilized thereto as will be further described below.

Since the beam of light 5 reflected at the glass-metal interface represents a continuous range of incident angles, a shift in the resonance angle caused by a change in the concentration of biomolecules at the metal surface may be detected by the detector unit 7. For a more comprehensive description of the above described biosensor system, including optical system, sensor unit, and liquid handling unit, it is referred to our aforementioned WO 90/05295.

The use of a biosensor system of the above outlined type for the purposes of the present invention will now be described. Fig. 2 schematically illustrates a part of a liquid handling block unit 9 comprising four flow cells 10, corresponding to flow cell 1 in Fig. 1. The flow cells 10 are, as in Fig. 1, defined by upwardly open channel parts 11 covered by a sensor plate 12, corresponding to sensor plate 2 in Fig. 1. In the figure is also illustrated an optointerface 13 for effecting optical contact between the sensor plate 12 and a prism corresponding to prism 4 in Fig. 1. The optointerface consists of a thin glass plate having elastic material pieces 14 on both sides with a refractive index matching that of the sensor plate 12. When the assembly of liquid handling block unit 9, sensor plate 12 and optointerface 13 is arranged in the biosensor system schematically illustrated in Fig. 1 (replacing flow channel 1 and sensor plate 2 therein), the wedge-shaped beam 5 will extend transversely across the flow cells, each flow cell corresponding to, e.g., one column of photodetectors in the detector matrix 7.

The gold-coated glass plate 12 has, as a specific example known per se in the art, a hydrophilic matrix of non-crosslinked carboxymethylated dextran covalently bonded to the gold film through an optically and biologically inert linker layer of long chain hydrocarbon. Ligands can be covalently immobilized to the dextran layer after

activation, e.g. by derivatization with N-hydroxy-succinimide (NHS), mediated by N-ethyl-N'-(dimethyl-aminopropyl)carbodiimide (EDC). The NHS-ester formed readily reacts with uncharged primary amino groups of the ligands to be coupled thereto.

In the flow cells 10 shown in Fig. 2 the sensing areas forming the top part of each flow cell may, for the purposes of the invention, in three of the flow cells 10 support ligands reactive with a respective cardiac analyte to be determined whereas the fourth flow cell preferably is used as a control or reference. For example, one flow cell may support a monoclonal antibody against creatine kinase MB (CK-MB), a second flow cell a monoclonal antibody against myoglobin, a third flow cell a monoclonal antibody against troponin I, and a fourth flow cell a monoclonal antibody against carbonic anhydrase III (CA III) as a control. Of course, more than four flow cells may be used if desired. Thus, for instance, a fifth flow cell may support streptokinase ligands for determining streptokinase antibodies in a sample. Optionally, a sixth flow cell may support a ligand for an additional cardiac infarction analyte.

For further details on the immobilization of ligands and reaction conditions for the coupling of the analytes to be determined, it is referred, in addition to the above mentioned WO 90/05303 and WO 90/05305, to the manual "Real-time Biospecific Interaction Analysis, A Guide to Methods and Applications" (1990), Pharmacia Biosensor AB, Uppsala, Sweden (the full disclosure of which is incorporated by reference herein).

A test of a blood plasma sample for the cardiac enzymes creatine kinase MB, myoglobin, and troponin I using the analytical system illustrated in Figs. 1 and 2 may be performed as follows. After removal of the blood cells from a blood sample, a defined volume of the plasma is introduced into the liquid handling unit 9 and evenly distributed among the four flow cells 10. When flowing through the flow cells the mentioned cardiac enzymes, if

present, will bind to the respective sensing surface supporting the proper ligand. This will cause a shift of the resonance angle, which shift is proportional to the amount of cardiac enzyme bound to the surface, as described above. Thereby the level of each analyte in the sample may be determined. To increase the resonance angle shift detected, the ligand bound analytes are preferably reacted with a secondary reagent in sandwich assay fashion. As already mentioned above, such a secondary reagent may optionally be labelled with an optically dense species to still more increase the shift. Alternatively, a tertiary reagent may be used. After the detection step, the sensor surface is regenerated by passing a regenerating agent, e.g. glycine buffer, or phosphoric, formic or hydrochloric acid (10-100 mM), through the cell. A whole such analytical procedure will take about 15 minutes.

Instead of using several flow cells as described above, a single flow cell may be used which has ligands for all the analytes of interest co-immobilized on the same sensing surface area. This embodiment will also be illustrated in the Example below.

When a patient with suspected AMI has been brought to the coronary care unit in possession of the biosensor apparatus described above at the patient's bedside, and it has not been possible to establish a safe diagnosis based upon symptoms and ECG, blood samples are taken regularly, say every 15 to 30 minutes, and successively analyzed in the described fashion. While already the first sample tested may exhibit such levels of one or more of the analytes tested for that an AMI may directly be diagnosed, a safe and reliable AMI diagnosis or exclusion, based upon the development with time of the analyte levels, will in the majority of cases be obtained within about no more than about two to three hours. In the case of a diagnosed AMI, the patient can then be treated with thrombolytics, i.e. streptokinase or tPA. Preferably, the test panel of the sensor unit also contains a sensing surface area for testing for streptokinase antibodies, and information about

the proper thrombolytic and dosage thereof to select may thereby be obtained at the same time, i.e. resulting in that the dose of streptokinase is adjusted or that tpA is selected rather than streptokinase.

The same bedside apparatus and test panel may then be used for monitoring the thrombolytic treatment to observe a reperfusion as soon as possible after it has taken place, so that the per se risky treatment may be stopped when no longer necessary or an alternative treatment may be introduced if reperfusion is not obtained in a reasonable time. Especially in such monitoring of thrombolytic treatment, relatively high levels of an analyte of interest, e.g. CK-MB, may be obtained. Thus, if a secondary reagent is used as described above, the dynamic range of the secondary response may not be sufficient for permitting the development of the peak value of a specific analyte to be monitored. The occurrence and exact monitoring of such a peak indicative of reperfusion may then be performed by instead measuring the primary response, i.e. the complex formation between the analyte and the immobilized ligand. Naturally, the above described procedure and method may also be used for monitoring the myocardium state during thorax surgery such that an AMI initiated during the surgery may be detected and treated before the thorax is closed.

In the following non-limiting example, the detection of CK-MB and myoglobin in a plasma sample in accordance with the present invention is described, using a commercial SPR-based biosensor instrument (BIAcore™) and sensing surface (Sensor Chip™ CM5) (both marketed by Pharmacia Biosensor AB, Uppsala, Sweden).

EXAMPLE

A. Co-immobilization of monoclonal antibodies on sensing surface

Immobilization of a monoclonal antibody specific for CK-MB and a monoclonal antibody specific for myoglobin was performed in the biosensor instrument in the following manner:

A continuous flow of HBS (10 mM Hepes buffer, 0.15 M NaCl, 3.4 mM EDTA, 0.05 % Tween), pH 7.4, over the sensing surface was maintained at 5 µl/min. A fraction of the carboxyl groups on the sensing surface was activated to form reactive N-hydroxysuccinimide esters by injecting into the instrument 35 µl of a solution containing 0.2 M 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 0.05 M N-hydroxysuccinimide (NHS) in water. 35 µl of the antibody solution containing 50 µg/ml of a monoclonal antibody specific for CK-MB (obtained from BiosPacific, Emeryville, California, U.S.A.) and 50 µg/ml of a monoclonal antibody specific for myoglobin (obtained from the Institute of General and Molecular Pathology, Tartu State University, Tartu, Estonia) in 10 mM sodium acetate, pH 5.0, were then injected. A buffer with a pH below the pI of the antibody will give a positive net charge of the protein, and at low ionic strength the antibodies will preconcentrate to the remaining negatively charged carboxyl groups on the surface via electrostatic attraction giving a high antibody concentration in the matrix. The preconcentration allows fast immobilization with low amount of antibodies. Remaining reactive ester groups were deactivated by injection of 35 µl of 1 M ethanolamine hydrochloride, pH 8.5. The sensorgram obtained is shown in Fig. 3 (response in resonance units, RU, plotted versus time in seconds). The response signal was evaluated at two levels: 20 seconds before the injection of EDC/NHS (A) and 9 minutes after the injection of ethanolamine (B). B minus A thus defines the immobilized amount of the two antibodies.

B. Analysis of plasma samples

The analysis of CK-MB and myoglobin at elevated levels in a plasma sample, using the sensing surface with co-immobilized antibodies prepared in section A above, was performed in the following manner:

A continuous flow of HBS (10 mM Hepes buffer, 0.15 M NaCl, 3.4 mM EDTA, 0.05 % Tween), pH 7.4, over the sensing surface was maintained at 5 µl/min. 35 µl of a plasma

sample containing CK-MB and myoglobin were injected into the instrument. 4 µl each of second antibodies specific for CK-MB and myoglobin, respectively, at a concentration of 100 µg/ml were then injected in sequence followed by 4 µl of 10 mM glycine-HCl, pH 2.5. The sensorgram obtained is shown in Fig. 4 (response in resonance units, RU, plotted versus time in seconds). The response signal was evaluated at four levels: 20 seconds before the injection of the sample (A), 20 seconds before the injection of the second antibody specific for CK-MB (B), 20 seconds before the injection of the second antibody specific for myoglobin (C), and 20 seconds before the injection of glycine-HCl (D). Thus, A defines the baseline, B minus A defines the plasma response, C minus B defines the specific response for CK-MB, and D minus C defines the specific response for myoglobin. The analysis time was 18 minutes.

The same procedure as described above was then performed for a plasma sample not containing CK-MB and a normal myoglobin level. The sensorgram obtained is shown in Fig. 5. As appears therefrom, no response was obtained when injecting the second antibody specific for CK-MB, whereas a weak response was obtained for the second antibody specific for myoglobin.

The invention is, of course, not restricted to the above specifically described embodiments, but many modifications and changes may be made without departing from the scope of the general inventive concept as defined in the subsequent claims.

CLAIMS

1. A method of determining myocardial infarction markers, comprising the steps of

(i) simultaneously determining from a first blood, serum or plasma sample from a patient at least two different analytes indicative of myocardial infarction by contacting in a flow cell or cells the sample with one or more sensor surface areas each supporting a different ligand or mixture of different ligands capable of specifically binding to a respective analyte, and, optionally after the additional binding of an analyte specific reagent or reagent complex to the ligand bound analytes, detecting any binding interaction of each analyte with its ligand as a consequential change of the physico-chemical characteristics of the sensor surface;

(ii) removing each bound analyte from its sensor surface bound ligand by passing a regenerating liquid through the flow cell;

(iii) repeating step (i) for at least a second blood, serum or plasma sample taken from the patient at a determined time interval from said first sample; and

(iv) determining from the results of steps (i) to (iii) the variation with time of said cardiac analytes.

2. A method according to claim 1, wherein said change of the physico-chemical characteristics of the sensor surface is a change in refractive index measured by evanescent wave spectroscopy.

3. A method according to claim 2, wherein said evanescent wave spectroscopy is surface plasmon resonance spectroscopy.

4. A method according to claim 1, 2 or 3, wherein the sample is contacted with a plurality of sensor surface areas, each supporting a different ligand.

5. A method according to claim 1, 2 or 3, wherein the sample is contacted with a single sensor surface area having different ligands co-immobilized thereto.

6. A method according to any one of claims 1 to 5, wherein at least three different cardiac analytes are determined.

7. A method according to any one of claims 1 to 6, which additionally comprises determining streptokinase antibodies in the sample by binding to a corresponding ligand immobilized to the or one sensor surface area.

8. A method according to any one of claims 1 to 7, wherein said cardiac analytes are selected from the group consisting of myoglobin; creatine kinase and its isoenzymes and isomers thereof, especially creatine kinase MB; myosin, especially the light chain thereof; tropomyosin; lactate dehydrogenase (LD); aspartate aminotransferase; and troponin C, T and I.

9. A method according to claim 8, wherein said cardiac analytes are selected from creatine kinase MB, myoglobin and troponin I.

10. A method according to any one of claims 1 to 9, which comprises determining at least one control analyte.

11. A method according to claim 10, wherein myoglobin is determined and carbonic anhydrase III is a control analyte.

12. Use of the method according to any one of claims 1 to 11 for diagnosing and/or excluding myocardial infarction.

13. Use of the method according to any one of claims 1 to 11 for the monitoring of treatment with thrombolytics.

14. A sensor means comprising, immobilized to one or more sensing areas thereof, either individually or in combination, at least two, preferably at least three different ligands, each ligand being capable of specifically binding to a respective analyte indicative of myocardial infarction, said sensor means being adapted for the detection of any analyte-ligand interaction as a consequential change of the physico-chemical characteristics of the sensing surface, and said ligand supporting surface areas being regeneratable after the coupling of analytes thereto.

15. A sensor means according to claim 14, which comprises a plurality of sensing areas, each area supporting a different ligand.

16. A sensor means according to claim 14, which comprises a single sensing area having different ligands co-immobilized thereto.

17. A sensor means according to any one of claims 14 to 16, wherein said ligands are selected from monoclonal antibodies against cardiac enzymes or proteins selected from the group consisting of myoglobin; creatine kinase and its isoenzymes and isomers thereof, especially creatine kinase MB; myosin, especially the light chain thereof; tropomyosin; lactate dehydrogenase (LD), aspartate aminotransferase, and troponin C, T and I; said ligands preferably being selected from the group consisting of creatine kinase MB, myoglobin and troponin I.

18. A sensor means according to any one of claims 14 to 17, which additionally comprises immobilized to a sensing area thereof a ligand capable of binding streptokinase antibodies.

19. A sensor means according to any one of claims 14 to 18, which additionally comprises immobilized to a sensing

area or areas thereof at least one ligand for a control analyte.

20. A sensor means according to any one of claims 14 to 19, which is adapted for surface plasmon resonance spectroscopy.

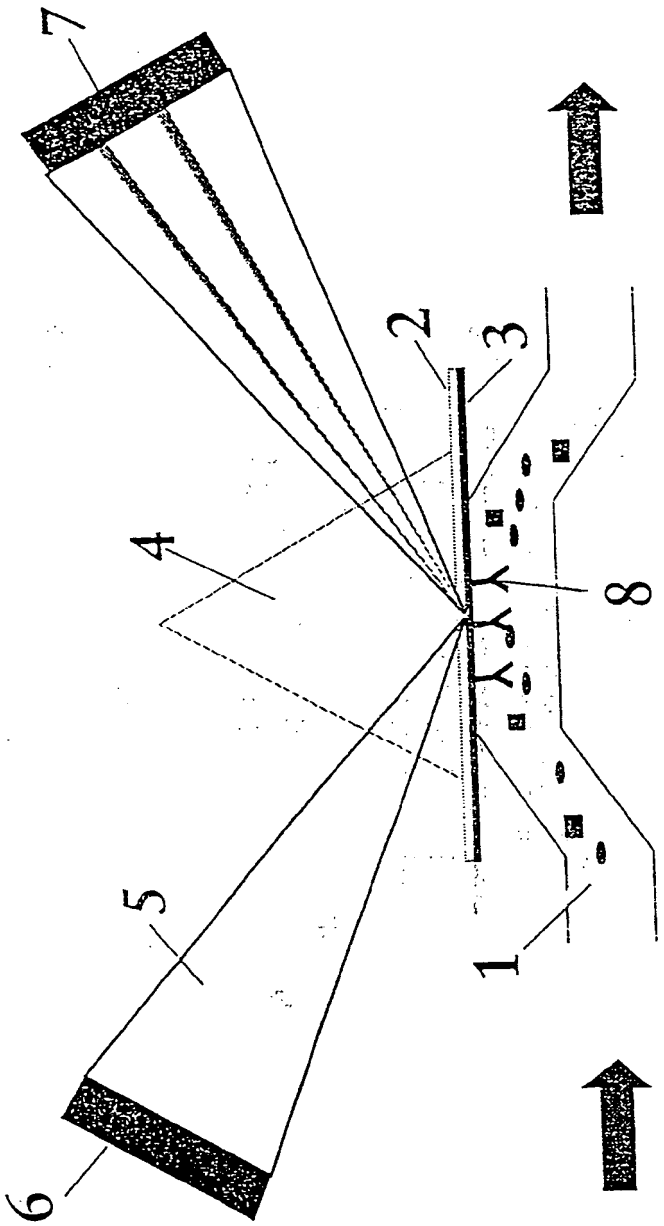

FIG.1

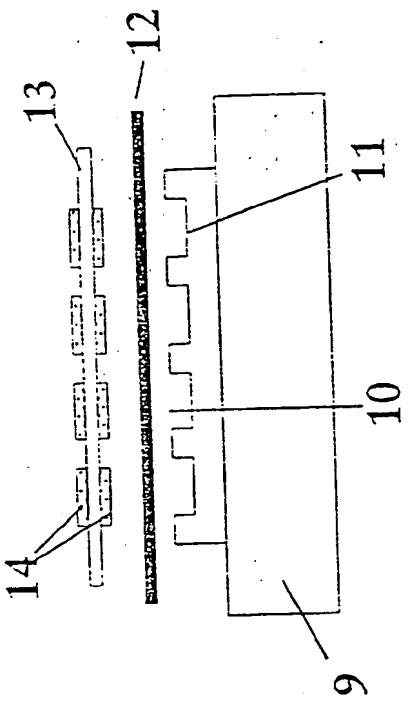

FIG. 2

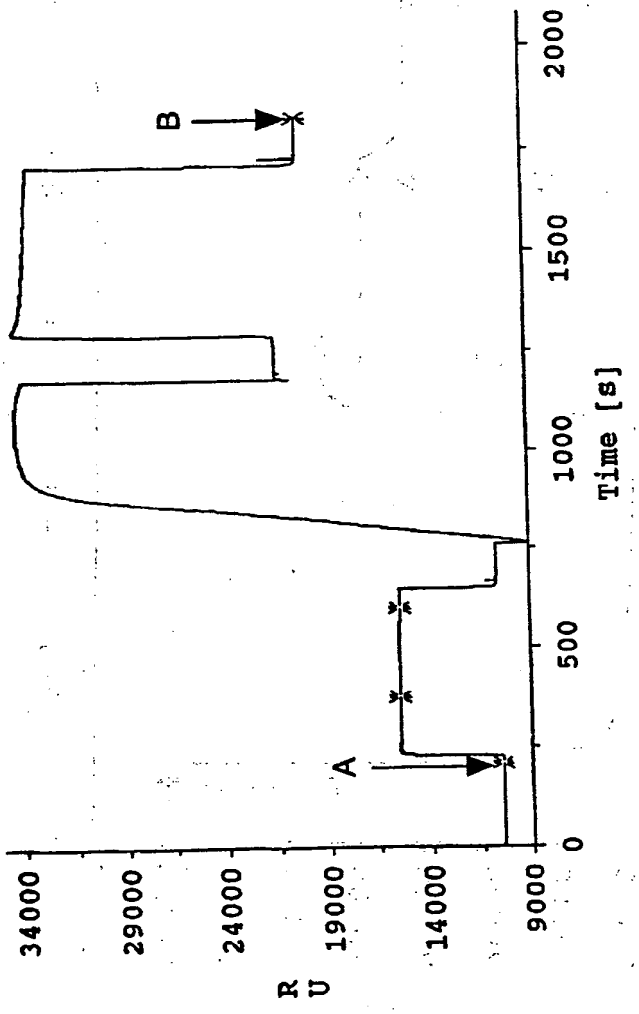

FIG. 3

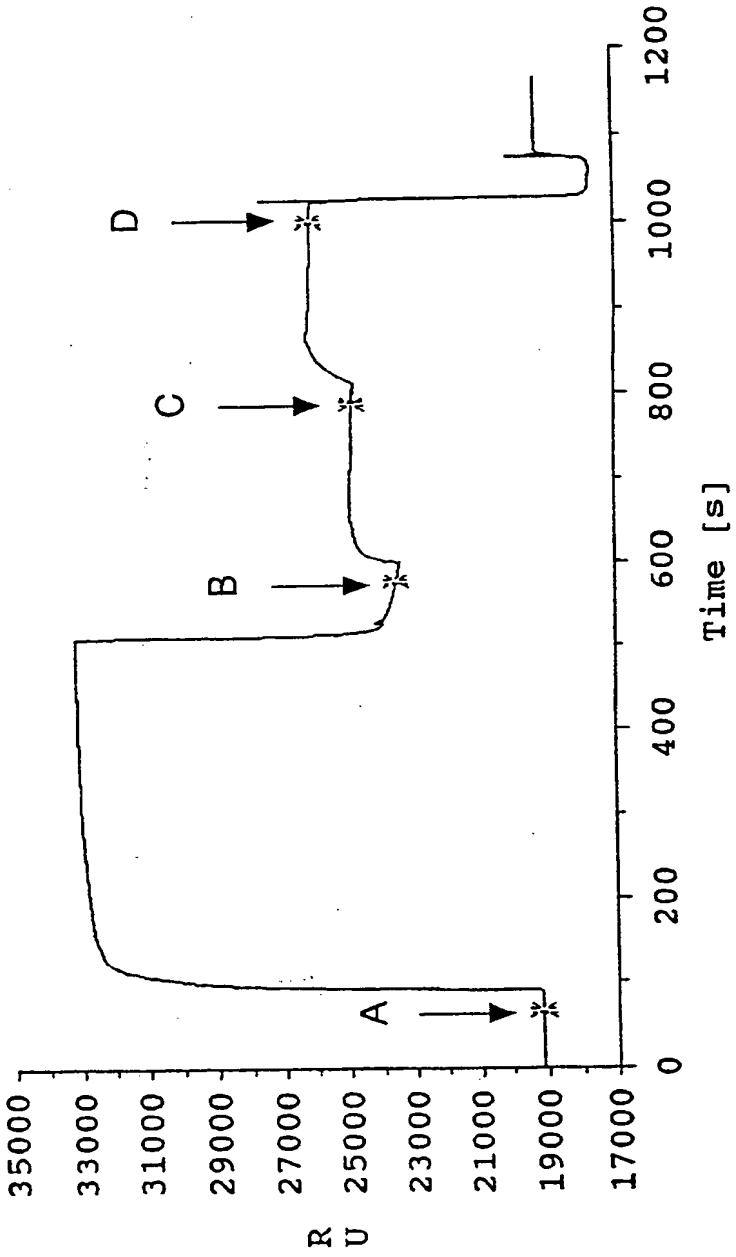

FIG. 4

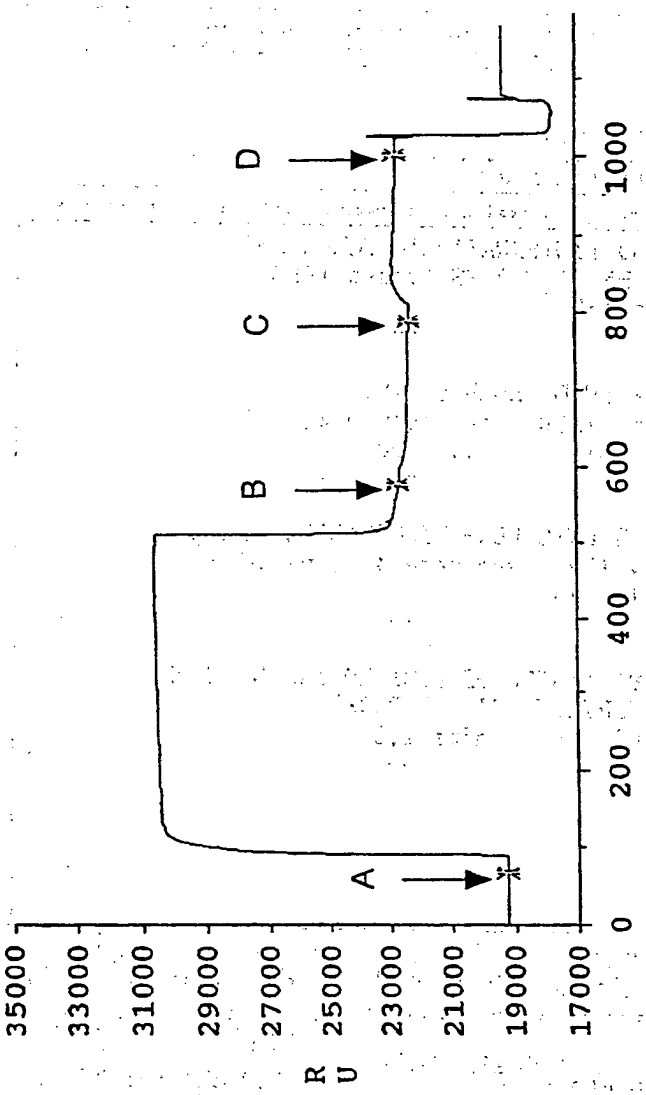

FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 92/00386

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: G 01 N 33/53, 33/543

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP, A2, 0384130 (INTERNATIONAL IMMUNOASSAY LABORATORIES, INC.) 29 August 1990, see claims 1-5 --	1,12
A	US, A, 4900662 (V.D. SHAH ET AL.) 13 February 1990, see column 4, line 35 - column 5, line 60 --	1
A	WO, A1, 9101498 (VIOCLONE BIOLOGICALS INC.) 7 February 1991, see page 4, line 32 - page 6, line 14 --	1-12
A	EP, A2, 0266881 (ASTROMED LIMITED INNOVATION CENTRE CAMBRIDGE SCIENCE PARK) 11 May 1988, see claims 1,8 --	1,2, 14

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
7th September 1992	1992 -09- 10
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Inga-Karin Petersson Inga-Karin Petersson

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	WO, A1, 9005305 (PHARMACIA AB) 17 May 1990, see the whole document --	14
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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/SE 92/00386

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 31/07/92 The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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		JP-A-	1054260	89-03-01
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		EP-A-	0442930	91-08-28
		JP-T-	4501606	92-03-19
		JP-T-	4501607	92-03-19
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